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# DIFFERENTIATING BONE OSTEONAL TURNOVER RATES BY DENSITY FRACTIONATION; VALIDATION USING THE BOMB $^{14}C$ ATMOSPHERIC PULSE

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**ABSTRACT.** The density (BSG) of bone increases, at the osteon scale, during lifetime aging within the bone. In addition, post-mortem diagenetic change due to microbial attack produces denser bioapatite. Thus, fractionation of finely powdered bone on the basis of density should not only enable younger and older populations of osteons to be separated but also make it possible to separate out a less diagenetically altered component. We show that the density fractionation method can be used as a tool to investigate the isotopic history within an individual's lifetime, both in recent and archaeological contexts, and we use the bomb $^{14}C$ atmospheric pulse for validating the method.

## INTRODUCTION

Human bones recovered from archaeological sites are an invaluable source for reconstructing paleodiet, paleoenvironment, and other archaeological information. Since remodeling of bone occurs throughout life, information corresponding to times of bone formation is therefore potentially available, provided that the appropriate fraction of bone can be selected. This might enable life-histories to be reconstructed. Such a method would be applicable to fresh as well as archaeological bone. We have developed a density fractionation method, modified from Bell et al. (2001), and here report results which demonstrate its effectiveness in the context of isotopic measurements. An additional benefit is an ability to distinguish bone material which has become hypermineralized through diagenetic action.

We show also that different fractions of human bone correspond to different ages of the individual's lifetime by measuring how the atmospheric nuclear bomb test radiocarbon pulse is incorporated into the bone collagen by diet during the subject's lifetime.

### The Specific Gravity of Living Bone

Bone density (specific gravity/BSG) depends on the degree of mineralization resulting from bone formation and maturation. The BSG is controlled by the precipitation of mineral salts, rather than collagen, displacing interstitial water (Figure 1). The BSG increases during lifetime maturation of the bone, and the spatial variation of BSG corresponds to bone packets of different "tissue age" (Lowenstam and Weiner 1989). Macroscopic bone exhibits a variety of growth structures of which the most typical, especially in cortical adult bone, is based on osteons. These functional units are generally axially aligned with diameters of about 150 μm (Fincham 1969). Remodeling involves the replacement of portions or groups of osteons with new, complete osteons. Microscopy of bone sections reveals new osteons as superimpositions; the greater mineralization of the older osteons is also revealed by electron back-scattering SEM. Thus, we expect a density difference between older and younger osteons.

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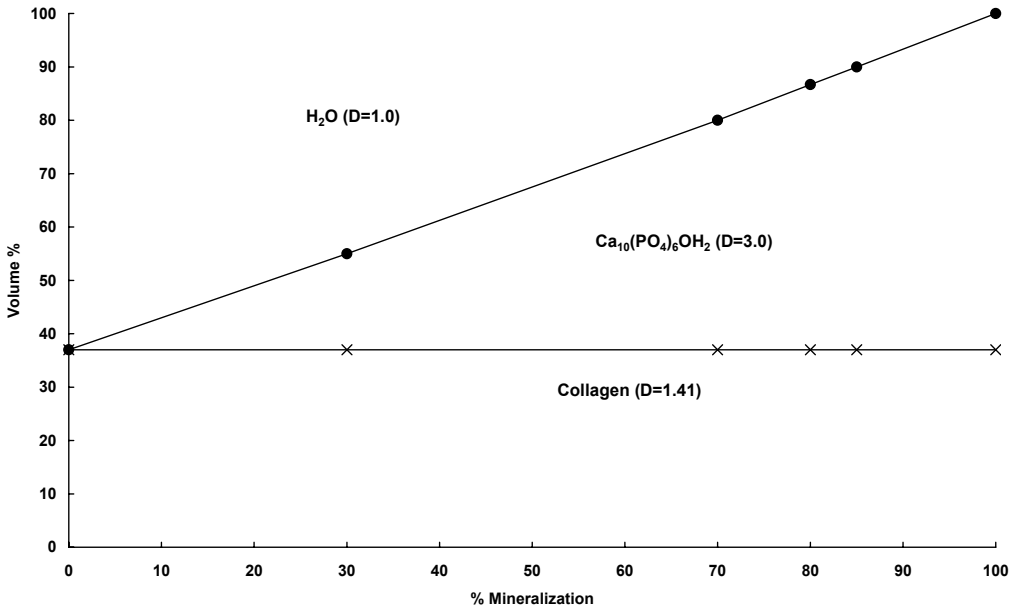


Figure 1 Relative proportion of main components of bone (mineral, collagen, and water) as a function of degree of mineralization. Redrawn from Lowenstam and Weiner (1989).

### Bone Turnover

The rate and mode of bone turnover is a vast subject; only the most relevant issues are treated here. In growing bone, the processes of resorption (by osteoclasts) and synthesis (by osteoblasts) occur in quite different locations. In mature bone, remodeling involves the continual replacement of existing bone, both the mineralized and the organic component. The fraction of bone remodeled per yr defines a turnover rate, which is known to vary between skeletal elements, to depend on applied chronic stresses, and to change with age. Estimates of the rate are in the region of 2–8% per yr for cortical bone for adults (Newman and Newman 1958; Frost 1969; Marshall et al. 1973; Stenhouse and Baxter 1979). One source of such estimates is to measure the extent to which the $^{14}C$ pulse, produced in the atmosphere in the 1960s by nuclear bomb testing and incorporated into the terrestrial biosphere, is taken up in human bone through the diet. We have used this method to verify that different fractions (separated by BSG), which are believed to represent different tissue ages, indeed do correspond to different retention times in bones based on variations in these fractions' $^{14}C$ bomb pulse.

## APPROACH

The development of the BSG method and its potential application to archaeology was first reported by Bell et al. (2001). Methodologically, our approach differs in the grinding technique and in the particle size distribution achieved. We show here that isotopic measurements can be made on different fractions which are consistent with expectation, especially the bulk value, and in particular, can show that different BSG fractions can correspond to different periods of bone formation in an individual.

Since bone is remodeled at an osteonal level of structure, the BSG separation must be carried out on particles that are small compared with an osteon. Therefore, after grinding (see under "Materials and Methods"), we checked the particle size distribution with a laser particle sizer. Fractionation by

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BSG separation is achieved by differential flotation in bromoform-methanol mixtures. (We note that we failed to achieve BSG separation on such a fine particle population using sodium polytungstate solutions, probably because of the propensity to flocculate in ionic media.)

We extracted collagen from the separated fractions and compared $\delta^{13}C$ and $\delta^{15}N$ measurements on the fractions with the bulk material to check consistency. Using archaeological bone, which is frequently diagenetically altered by microbial action, we separated lifetime fraction and diagenetic fraction by BSG. Much of the archaeological bone are present in "hypermineralized" form, with loss of its original micromorphology. This altered material, which chemically still corresponds to hydroxyapatite, has a substantially greater BSG, and so can be efficiently removed from unaltered bone by BSG separation. We also made $^{14}C$ AMS measurements on collagen from the separated BSG fractions from modern bone, so that the effect of the post-1960s atmospheric $^{14}C$ pulse could be used to compare the relative times of bone formation. This can be used as a direct test that the different BSG fractions correspond to different tissue ages within the same individual.

## MATERIALS AND METHODS

Samples were taken from archaeological bones from 2 sites (Greyfriars, Oxford, UK, Late Saxon; Repton, UK, Middle Saxon). Histological examination showed all archaeological bone had some degree of diagenetic alteration. Modern human bone was obtained from forensic and orthopedic studies; these samples were used to test variation in tissue ages by $^{14}C$ analysis. Modern bone samples were defatted by sequential rinses in chloroform and methanol mixture (2:1), then water. Archaeological bone samples were cleaned by shotblasting. All bone samples were then milled to a size distribution in which over 90% of particles could be calculated as comprising material from 1 osteon only, where mean osteon diameter is assumed to be 150 μm (Fincham 1969). The size distribution was measured by a Coulter Laser Particle Size Analyser (Beckham Coulter LS 230 laser granulometer) within the range of 0.04–2000 μm and confirmed by optical microscopy. The milling was carried out sequentially using a Disc Mill (TEMA, Machinery, Ltd Banbury, Oxon, Laboratory Disc mill) and Micronizing Mill (McCRONE Micronizing mill, McCrone Research Associates Ltd.). In general, it is necessary to have >90% of particles smaller than 9.0 μm in diameter and we note that adequate milling is critical to the success of the method. Fractions were then collected by centrifugation after differential flotation in a sequence of increasing specific gravity mixtures of bromoform and methanol (8–10 ml was added to centrifuge tube) with densities of 1.8, 2.0, 2.1, 2.2, 2.4, and 2.6 $g/cm^3$.

Following washing in methanol, distilled water, and lyophilization, collagen was extracted by decalcification in 0.5 M aqueous HCl with gelatinization of the insoluble material at pH 3 for 48 hr at 75 °C and subsequent lyophilization of the filtrate. A 3-mg aliquot of the product was then combusted in a continuous-flow isotope ratio mass spectrometer (Carlo Erba carbon and nitrogen elemental analyzer coupled to a Europa Geo 20/20 mass spectrometer) to $CO_2$, $H_2O$, and $N_2$ and measured for C/N, $\delta^{13}C$, and $\delta^{15}N$. The GC-purified $CO_2$ was retained for $^{14}C$ measurement by AMS using standard procedures (Hedges et al. 1989; van Klinken and Hedges 1998).

## RESULTS

In Table 1, we provide the source, histological condition, and other information on samples selected for this study.

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Table 1 Samples used in this study.

| Abbreviated sample ID | Site | Sample ID | Bone type | Histological index [a] | Date of birth | Date of sampling |
|---|---|---|---|---|---|---|
| GREY1 | Greyfriars | OX69B II4 | Femoral shaft | 3~4 | — | — |
| GREY2 | Greyfriars | OX69B II2-3 | Femoral shaft | 3~4 | — | — |
| GREY3 | Greyfriars | OX69B IV36 | Femoral shaft | 3~4 | — | — |
| REP1 | Repton | R828 6L | Femoral shaft | 0~1 | — | — |
| REP2 | Repton | R828 718NW14R | Femoral shaft | 0~1 | — | — |
| REP3 | Repton | R828 718NW43R | Femoral shaft | 0~1 | — | — |
| FOR1 | Forensic sample | P 10321 | Cortical femur | — | 1935 | 1977 |
| FOR2 | Forensic sample | P 10323 | Cortical femur | — | 1947 | 1966 |
| NOC2 | Orthopaedic sample | NOC2 | Proximal tibia | — | 1915 | 1998 |
| NOC11 | Orthopaedic sample | NOC11 | Femoral head | — | 1920 | 1999 |
| NOCX23 | Orthopaedic sample | NOCX23 | Tibia/femur (compact bone) | — | 1964 | 1999 |
| NOCX24 | Orthopaedic sample | NOCX24 | Proximal tibia | — | 1915 | 1999 |

[a]Histological Index scored 0 to 5, where 5 implies very well-preserved bone and 0 indicates poorly preserved bone with hardly any original features (Hedges et al. 1995).

### BSG Distributions

The BSG range for fresh bone is rather small and only just sufficient to separate the different minority fractions. We were able to recover sufficient amounts of material in various BSG fractions for our analysis (see Figure 2). However, there is probably scope for improving the method to achieve better resolution. Well-preserved archaeological bone gives a somewhat wider distribution, which is not accounted for by the small variation in collagen content. For archaeological bone where there is a substantial fraction that has been microbially altered (i.e. for bones whose micromorphological structure has largely disappeared as assessed by visible microscopy of thick sections), the BSG distribution tends to an almost bimodal form (see Figure 2). In this case, the heaviest fractions contain relatively little collagen, presumably because of consumption by microbes.

### Stable Isotope Results and $^{14}C$ Results (Fresh Bone Only)

Table 2 presents $\delta^{13}C$ and $\delta^{15}N$ for each BSG fraction and the bulk source of bone. Also, collagen yields (%) and C/N ratios were used to indicate the integrity of the extracted collagen. Generally, for modern bones, collagen yield (%) is about 20%, while in archaeological bone >1% is normally accepted. The C:N ratio, which is derived from C and N content of the extracted "collagen," ranged from 3.2 to 3.6, within the accepted range of 2.9 to 3.6 (Hedges and van Klinken 1992; van Klinken 1999).

BSG fractions from modern bone were analyzed for their bomb carbon content (Table 3). The $^{14}C$ content is reported as % modern and the results are discussed in the following section.

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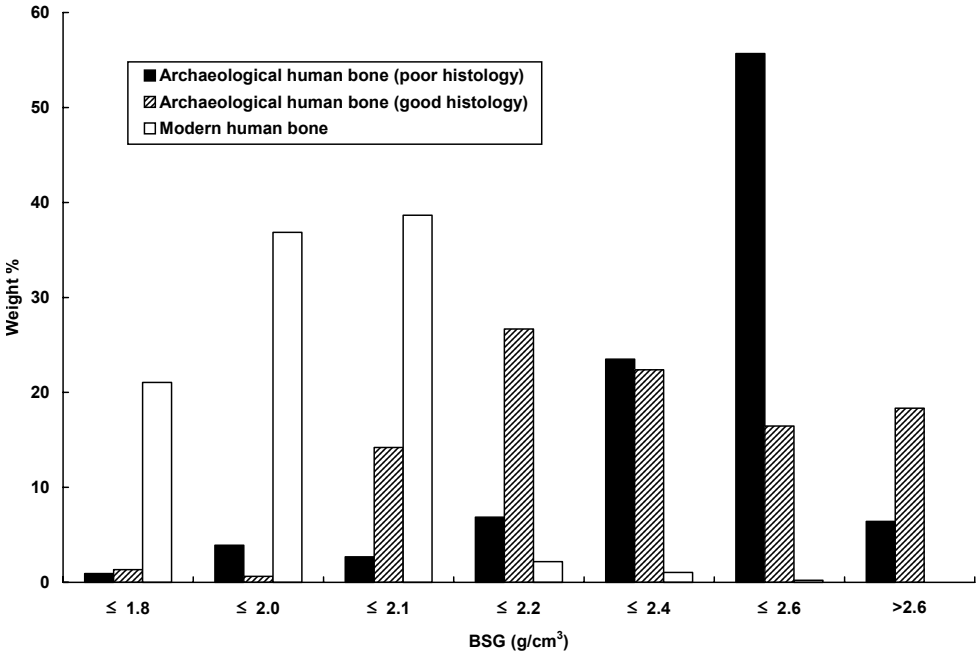


Figure 2 BSG distribution histogram of modern and archaeological human bone. In correlation to Table 1: Archaeological human bone (poor histology): REP1, REP2, REP3; archaeological human bone (good histology): GREY1, GREY2, GREY3; modern human bone: FOR1, FOR2, NOC2, NOC11, NOCX23, NOCX24.

Table 2 Stable isotope analysis results.

| BSG Fraction of the sample[a] | $\delta^{13}C$ (‰)[b] | $\delta^{15}N$ (‰)[b] | C/N | Collagen yield (%)[c] |
|---|---|---|---|---|
| GREY1-C | –18.2 | 12.6 | 3.3 | 13.6 |
| GREY1-D | –18.3 | 12.4 | 3.3 | 12.3 |
| GREY1-E | –18.4 | 12.6 | 3.3 | 8.4 |
| GREY1-F | –18.8 | 12.6 | 3.4 | 2.6 |
| GREY1 bulk | –18.3 | 12.6 | 3.3 | 12.2 |
| GREY2-C | –20.0 | 13.0 | 3.3 | 16.1 |
| GREY2-D | –18.9 | 12.6 | 3.3 | 10.4 |
| GREY2-E | –18.9 | 12.8 | 3.3 | 7.7 |
| GREY2 bulk | –18.9 | 12.7 | 3.3 | 11.1 |
| GREY3-C | –19.8 | 10.7 | 3.3 | 7.2 |
| GREY3-D | –19.7 | 10.4 | 3.3 | 11.7 |
| GREY3-E | –19.5 | 10.6 | 3.3 | 7.8 |
| GREY3-F | –19.8 | 11.0 | 3.4 | 3.9 |
| GREY3 bulk | –19.6 | 10.5 | 3.3 | 12.2 |
| REP1-E | –19.4 | 11.3 | 3.4 | 3.2 |
| REP1-F | –19.9 | 10.8 | 3.4 | 6.0 |
| REP1 bulk | –19.4 | 10.9 | 3.3 | 7.1 |

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Table 2 Stable isotope analysis results. *(Continued)*

| BSG Fraction of the sample[a] | $\delta^{13}C$ (‰)[b] | $\delta^{15}N$ (‰)[b] | C/N | Collagen yield (%)[c] |
|---|---|---|---|---|
| REP2-D | –19.9 | 11.0 | 3.4 | 5.1 |
| REP2-E | –20.5 | 11.1 | 3.6 | 1.6 |
| REP2-F | –20.4 | 10.9 | 3.6 | 1.3 |
| REP2 bulk | –19.8 | 11.1 | 3.3 | 7.5 |
| REP3-D | –20.2 | 11.4 | 3.5 | 2.6 |
| REP3-E | –19.8 | 11.1 | 3.4 | 5.7 |
| FOR1-B | –16.9 | 11.9 | 3.4 | 20.8 |
| FOR1-E+F | –17.2 | 11.0 | 3.3 | 21.7 |
| FOR1 bulk | –17.3 | — | — | — |
| FOR2-B | –18.7 | 11.6 | 3.5 | 23.1 |
| FOR2-C+F | –16.9 | 11.0 | 3.3 | — |
| FOR2 bulk | –18.4 | — | — | — |
| NOC2-A | –19.0 | 12.2 | 3.4 | 33.0 |
| NOC2-B | –19.3 | 12.2 | 3.3 | 21.2 |
| NOC2-C | –18.9 | 12.4 | 3.2 | 16.5 |
| NOC2 bulk | –18.9 | 12.4 | 3.2 | 20.3 |
| NOC11-B | –19.8 | 12.1 | 3.5 | 25.8 |
| NOC11-C | –19.1 | 12.3 | 3.3 | 25.2 |
| NOC11 bulk | –19.1 | 12.2 | 3.3 | 16.7 |
| NOCX23-A | –20.2 | 11.0 | 3.5 | — |
| NOCX23-B | –19.7 | 10.9 | 3.3 | 28.8 |
| NOCX23-C | –19.3 | 11.0 | 3.2 | 23.8 |
| NOCX23-D | –19.0 | 11.5 | 3.2 | 25.1 |
| NOCX23 bulk | –19.3 | 11.1 | 3.3 | 24.3 |
| NOCX24-A | –19.5 | 12.6 | 3.4 | 36.4 |
| NOCX24-B | –20.4 | 11.9 | 3.5 | 33.3 |
| NOCX24-C | –19.9 | 12.2 | 3.2 | 11.2 |
| NOCX24 bulk | –19.3 | 12.9 | 3.2 | 29.9 |

[a]Sample code: the specific gravity of each sample is coded as follows: A (≤1.8), B (1.8~2.0), C (2.0~2.1), D (2.1~2.2), E (2.2~2.4), F (2.4~2.6), G (>2.6)

[b]$\delta^{13}C$ and $\delta^{15}N$ values are expressed as ‰ relative to VPDB standard and AIR standard respectively.

[c]Collagen yield are calculated from extracted collagen amount versus amount of bone initially used.

Note that isotopic data were available only from BSG fractions containing more than 50 mg bone.

Table 3 $^{14}C$ results of modern BSG fractions.

| Sample index[a] | OxDate (pMC) | $\delta^{13}C$ (‰) | OxA |
|---|---|---|---|
| FOR1-B | 107.0 ± 0.3 | –16.9 | OxA-X-2021-33 |
| FOR1- E+F | 102.2 ± 0.3 | –17.2 | OxA-X-2022-37 |
| FOR1 bulk | 110.2 ± 0.4 | –17.3 | OxA-9592 |

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Table 3 $^{14}C$ results of modern BSG fractions. *(Continued)*

| Sample index[a] | OxDate (pMC) | $\delta^{13}C$ (‰) | OxA |
|---|---|---|---|
| FOR2-B | 124.3 ± 0.3 | –18.7 | OxA-X-2021-34 |
| FOR2-C+ F | 103.6 ± 0.3 | –16.9 | OxA-X-2022-39 |
| FOR2 bulk | 126.0 ± 0.5 | –18.4 | OxA-9383 |
| NOC2-A | 110.3 ± 0.3 | –19.0 | OxA-X-2042-10 |
| NOC2-B | 111.5 ± 0.3 | –19.3 | OxA-X-2042-11 |
| NOC2-C | 114.5 ± 0.3 | –18.9 | OxA-X-2042-12 |
| NOC2 bulk | 113.5 ± 0.3 | –18.9 | OxA-X-2042-09 |
| NOC11-B | 110.2 ± 0.3 | –19.8 | OxA-X-2042-24 |
| NOC11-C | 109.0 ± 0.3 | –19.1 | OxA-X-2042-25 |
| NOC11 bulk | 110.4 ± 0.3 | –19.1 | OxA-X-2042-22 |
| NOCX23-A | 110.5 ± 0.3 | –20.2 | OxA-X-2042-14 |
| NOCX23-B | 110.6 ± 0.3 | –19.7 | OxA-X-2042-15 |
| NOCX23-C | 112.8 ± 0.4 | –19.3 | OxA-X-2042-16 |
| NOCX23-D | 119.2 ± 0.4 | –19.0 | OxA-X-2042-17 |
| NOCX23 bulk | 120.3 ± 0.3 | –19.3 | OxA-X-2042-13 |
| NOCX24-A | 110.0 ± 0.3 | –19.5 | OxA-X-2042-19 |
| NOCX24-B | 107.7 ± 0.3 | –20.4 | OxA-X-2042-20 |
| NOCX24-C | 111.6 ± 0.3 | –19.9 | OxA-X-2042-21 |

[a]Density: A (≤1.8), B (1.8~2.0), C (2.0~2.1), D (2.1~2.2), E (2.2~2.4), F (2.4~2.6), G (>2.6).

## DISCUSSION

It is important to demonstrate that the complicated processes of grinding, fractionating, and collagen preparation from very fine powder do not incorporate contaminating material. The measurements of collagen (%, C/N, and isotopic composition) from each fraction are generally in accord with that from the "bulk" (unfractionated) bone. In particular, there is no significant change in the isotopic composition between the fractions for samples NOC2; NOC11; NOCX23; NOCX24; GREY1,2,3; REP1,2,3. (i.e. the variance within the delta values is consistent with the errors expected from replicate measurements); this is not the case for FOR1 and FOR2, where a change in dietary history may be indicated.

$^{14}C$ analysis of modern bulk-bone and fractions tested the assumption that different density fractions were metabolized at different times within the subjects lifespan. Figure 3 shows how the lifetimes of 6 subjects are related to the timeline for the northern atmosphere $^{14}C$ bomb pulse (Goodsite et al. 2001; Levin and Hesshaimer 2000), and, therefore, approximately to the $^{14}C$ content of terrestrial diet available to the subjects during that timespan. The bulk determinations are roughly consistent with previously published observations (Stenhouse and Baxter 1979; Geyh 2001) for collagen.

When $^{14}C$ values for different fractions within the same individual are compared (Table 3), we note that the lighter BSG component consistently gives $^{14}C$ values which correspond to collagen synthesis at a later period along the bomb curve. This is most obvious for samples FOR1 and FOR2, for which the peak of the bomb pulse is later in life, but at an age where bone turnover is still quite active. For sample NOCX23, the bomb pulse occurs very early in life, so that synthesis at a later period of life (the lightest fractions A & B) correspond to relatively low atmospheric excess $^{14}C$. Thus, the relationship between $^{14}C$ value and BSG fraction is opposite to that of FOR1 and FOR2.

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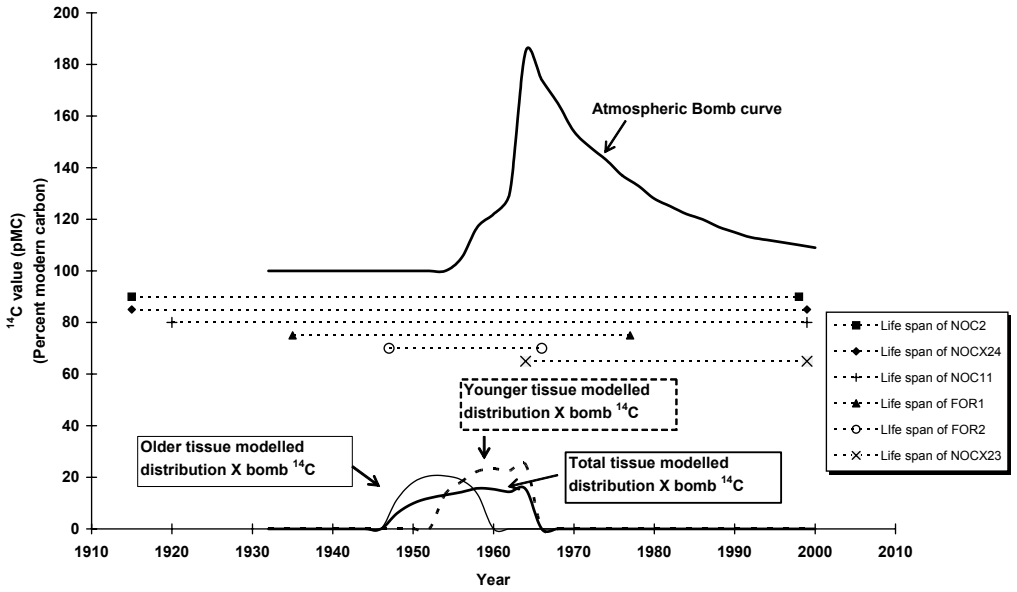


Figure 3 Relationship between the $^{14}$C dates and different BSG fractions. The lifespans of the different individuals measured for bone collagen $^{14}$C content are shown in relation to the timing of the expected $^{14}$C content of their diet. The lower part of the figure shows how an abitrary but feasible model of collagen turnover with age (using the lifespan of FOR2 as an example) can be used to calculate an expected collagen radiocarbon content in different components of the tissue. The turnover model results in an essentially parabolic distribution of tissue age; this distribution, when weighted by the dietary $^{14}$C content, gives the curves shown. The more mineralized, higher BSG fraction is modeled as a distribution of tissue age of surviving tissue from deposition during the first 75% of the individual's lifespan, while the less mineralized, lower BSG fraction is modeled as a distribution of surviving deposition during the last 75% of the individual's lifespan. The older (earlier) tissue remains approximately parabolic, since the bomb curve weighting is mainly constant, while the bulk and younger tissue fractions show $^{14}$C contents which are weighted towards higher values.

For samples NOC2, NOC11, and NOCX24, all with similar date of birth and age-at-sampling (of >83 yr), the bomb pulse occurs after a biological age of 45, when turnover rate is small. In these cases, there is little apparent difference between the light and heavy fractions, all of which contain roughly 25% of the total integrated bomb-produced $^{14}$C. If the light density fractions in these cases do correspond to recent synthesis (i.e. within the 10–20 yr before death), the value of the bomb curve at this point would not be differentiated from the measured BSG fractions synthesized earlier in life. Thus, while the results from these different individual samples are generally similar, it would appear that the bomb curve cannot usefully distinguish between early and later synthesized collagen in this particular situation.

We also note some inconsistencies in the $^{14}$C values. These are well outside analytical error, but perhaps record the presence of impurities (such as faster turning-over components from the bone). However, while the results are not entirely error-free, they are unequivocal in showing that the different fractions from a single bone sample give $^{14}$C values in qualitative agreement with their "tissue age." To obtain quantitative agreement presupposes a model of tissue turnover, which could only be constructed from a much larger data set. In principle, this method offers a way to investigate some points of the distribution of dates when surviving osteons are laid down in different bones in an individual (providing the bomb curve can be suitably involved).

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## CONCLUSION

We present evidence here for the validity of a method we have developed to fractionate bone into components of different "tissue age." This can be applied both to archaeological and fresh bone and enables stable isotopic analyses of carbon and nitrogen to be made of the bone collagen (and possibly other components). In particular, we have shown through $^{14}C$ dating of the collagen that the tissue age of the bone fractions can be qualitatively compared against models of bone turnover using the $^{14}C$ pulse of the atmospheric bomb curve. The comparison shows, at least for individuals whose age at death is 50 or less, that the lighter fractions contain collagen which was synthesized later in life. We consider the method to have application in investigating tissue age differences in relation to questions of bone physiology, as well as being applicable to studying archaeological material. A side advantage of the method is that diagenetically altered (hypermineralized) bone can be removed by this approach, allowing analysis of biogenic material; this may be its main use in archaeological and paleontological contexts. However, it provides a basis for the validity of measurements which aim to show lifetime changes in individuals as recorded in isotopic or other observations of bone fractions with different ages. We stress that these are initial results and further work should improve the methodology. In particular, the relationship between tissue age and each bone density fraction requires better resolution and clarification.

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